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6 MOLECULAR COMPOSITION INFLUENCES COLLAGEN GEL MECHANICS

COLLAGEN fibrils are the main structural element of connective tissues. In many tissues, these fibrils contain two fibrillar collagens (types I and V) in a ratio that changes during tissue development, regeneration, and various diseases. Here we investigate the influence of collagen composition on the structure and rheology of networks of purified collagen I and V, combining fluorescence and atomic force microscopy, turbidimetry, and rheometry. We demonstrate that the network stiffness strongly decreases with increasing collagen V content, even though the network structure does not substantially change. We compared the rheological data with theoretical models for rigid polymers and found that the elasticity is dominated by nonaffine deformations. There is no analytical theory describing this regime, hampering a quantitative interpretation of the influence of collagen V. Our findings are relevant for understanding molecular origins of tissue biomechanics and for guiding rational design of collagenous biomaterials for biomedical applications.

I.K. Piechocka, A.S.G. van Oosten, R.G.M. Breuls, G.H. Koenderink
Rheology of heterotypic collagen networks
Biomacromolecules **12**: 2797-805 (2011)

6.1 INTRODUCTION

Collagen is the main constituent of the extracellular matrix (ECM) of animal connective tissues [36]. Collagen forms elastic networks of fibrils that confer superior tensile strength. Moreover, the mechanical properties of collagen influence the behavior of cells within tissues [19, 115]. The biophysical mechanisms underlying the remarkable tensile strength of collagen have been studied extensively using fibrils isolated from tissue or reconstituted from purified collagen. Collagen fibers are rather stiff polymers with a Young's modulus in the range of 1-800 MPa in the hydrated state [273, 321, 105, 296, 271]. They form viscoelastic networks with elastic shear moduli in the range of 1-200 Pa, dependent on pH, ionic strength, and temperature [297, 156, 322, 282, 283, 243, 323, 8]. When strained, collagen gels tend to stiffen, which may serve as a mechanism to protect tissues against excessive deformation [156, 294, 285, 282, 283, 8].

Previous biophysical research has largely focused on networks of purified collagen I, the most abundant type of collagen in non-cartilaginous tissues. However, *in vivo* collagen I usually forms heterotypic fibrils together with collagen type II [122], III [251, 168, 287], or V [26, 186, 29, 185]. This co-assembly is thought to provide a mechanism for regulating fibril diameter. This view is mainly based on studies of collagen I and V, which form heterotypic fibrils whose diameter decreases with increasing collagen V content [86, 26, 3, 28, 56, 206]. In tissues, a similar inverse dependence of fibril diameter on collagen V content is observed. In most adult tissues, collagen fibrils contain only 2-5% collagen V and have a broad distribution of diameters in the range of 40-200 nm [202]. In the cornea, however, heterotypic fibrils contain 15-25% collagen V and have a uniform diameter of only 25-34 nm [202, 27, 139]. Several human connective tissue disorders such as Ehlers-Danlos syndrome are associated with increased collagen V content, indicating that a correct stoichiometry of collagen I and V is critical for normal tissue [56, 197, 7, 308]. Moreover, several other pathological conditions such as tumors, atherosclerotic plaques, and scars are also associated with increased levels of collagen V [35].

The effect of collagen V on the mechanical behavior of collagenous materials is unknown. The aim of our work was to study this effect in a model system of purified collagen I and V. The formation process of the heterotypic fibrils is relatively well understood. The building block of collagen fibrils is the *tropocollagen* molecule, a coiled triple helix composed of three α -chains. At 37°C and under appropriate buffer conditions (neutral pH, appropriate ionic strength, and presence of phosphate), purified tropocollagen spontaneously assembles into fibrils [317, 315]. Specific, non-covalent interactions lead to a precise axial stagger of each molecule by one-quarter of its length relative to its neighbor, giving the fibers a banding pattern with a periodicity of 67 nm in electron microscopy [230, 225]. This periodicity remains the same when collagen V is present [29, 3, 56, 184]. Several mechanisms have been proposed to account for diameter regulation by collagen V. Collagen is first synthesized as procollagen, which is enzymatically processed to collagen by cleavage of N- and C-terminal propeptides. In contrast to collagen I, collagen V retains a long (~ 17 nm) part of its N-propeptide. The collagen V helix is buried inside the

fibrils, but the N-propeptide extends outward through the gap zones of the quarter-staggered array. The exposed domains may limit lateral growth of fibrils by steric or electrostatic hindrance of monomer addition [28, 184, 56]. Yet, the diameter-limiting effect is still partly present when the N-propeptide is removed with pepsin, suggesting an influence of the collagen V helix on diameter regulation [3, 28, 56, 206]. The longer length of the type V helix compared to the type I helix (321 nm versus 299 nm) could make molecular packing less regular [272], or the larger amount of glycosylated hydroxylysine residues in collagen V may limit lateral growth [206].

In this chapter rheological measurements on collagen networks reconstituted from purified type I and V collagen will be presented. We varied the total collagen concentration and the composition of the networks. The microstructure of the pure and hybrid collagen networks was examined by fluorescence microscopy and the morphology of the fibrils by atomic force microscopy (AFM) and turbidimetry. We compare the rheological measurements with theoretical models of stiff polymers in an effort to uncover the physical origin of collagen V's influence on the rheology of hybrid collagen networks.

6.2 SAMPLE PREPARATION

Rat tail collagen I in 0.02 N acetic acid solution (with telopeptides) was purchased from BD Biosciences (Franklin Lakes, NJ). Lyophilized collagen V from human placenta was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.02 M acetic acid at 4 °C for 24 hours. This collagen V was pepsin-treated and thus lacked telopeptides, consistent with most prior studies of collagen structure and cell physiology in the presence of collagen V. Hybrid collagen networks were prepared from mixed stock solutions having collagen V contents of 0, 10, 20, 50, 80, and 100 wt%. Samples with a final volume of 300 μ l and total collagen concentration of 0.5-5 mg/ml were prepared on ice by diluting collagen with 30 μ l Minimal Essential Medium (MEM 10X, Gibco BRL, Paisley, Scotland) and water, and adjusting the pH to 7.3 with sodium hydroxide (Sigma-Aldrich) within 4 minutes to prevent premature polymerization [216]. MEM has a near-physiological ionic strength and pH and contains 1 mM phosphate, thus promoting formation of fibrils with a native D-banding pattern, as verified by AFM imaging.

For fluorescence microscopy, collagen was mixed on ice in a molar ratio of 7:1 with CNA35 [162], a bacterial collagen-binding protein with molecular weight of 35 kDa [261]. The CNA35 protein was labeled with Oregon Green and was a kind gift from dr. M. Merkx (TU Eindhoven, Netherlands). We checked by confocal microscopy on collagen networks with varying collagen/CNA35 molar ratio that the CNA35 protein did not affect collagen network structure. Moreover, collagen networks with CNA35 looked identical to unlabeled networks in bright field microscopy. The collagen solution was gelled at 37 °C in a glass microchamber, which was sealed to prevent solvent evaporation and imaged as described in Chapter 2.

6.3 RESULTS

6.3.1 RHEOLOGY OF MIXED COLLAGEN I/V NETWORKS

Hybrid networks of collagen type I and V were polymerized between the plates of a rheometer as described in Chapter 2, using a steel cone (40 mm diameter, 1 degree angle, 49 μm truncation) and bottom plate. Rheology results were the same in parallel plate geometries with various gap sizes, indicating no-slip conditions. The time dependence of the shear moduli during polymerization is shown in Fig. 6.1 A, for samples with a fixed total collagen concentration of 2 mg/ml but varying collagen V content. For all compositions studied, the shear moduli began to increase immediately after initiation of collagen polymerization by heating to 37°C, with no detectable lag phase. From the onset, the elastic modulus was larger than the viscous modulus (inset of Fig. 6.1 A), suggesting that a space-spanning, elastic network was formed within 30 s. Polymerization was also evident from an immediate rise in gel turbidity (Fig. 6.1 B). The elastic modulus continued to rise slowly for at least 15 hours, but after 3 hours the increase was less than 10% over a 12 hour window. Therefore we decided to perform all rheological tests after 3 hours of polymerization (arrow in inset of Figure 6.1 A).

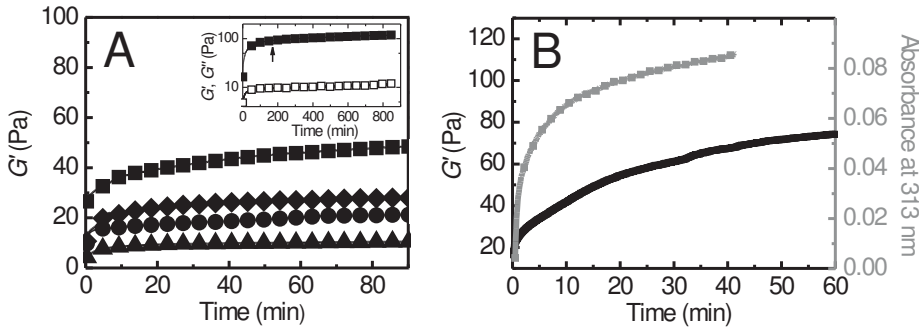


FIGURE 6.1. Polymerization behavior of collagen networks. (A) Time dependence of the elastic modulus of collagen solutions heated to 37°C inside the rheometer with varying proportions of type I and V and a fixed total collagen concentration of 2 mg/ml. Symbols denote weight/weight ratios of collagen I to collagen V: 100/0 (squares), 90/10 (diamonds), 80/20 (circles) and 0/100 (triangles). *Inset*: Elastic (solid squares) and viscous (open squares) shear moduli for 3.5 mg/ml collagen type I gel. (B) Network polymerization monitored by rheology (black line) and turbidimetry (red line) for 3.5 mg/ml collagen type I gel.

Incorporation of collagen V substantially decreased the final gel stiffness. Pure collagen I networks of 2 mg/ml had an average stiffness of 45 Pa while pure collagen V networks had a 10-fold lower stiffness of 4 Pa ($p < 0.01$). The mixed networks had intermediate values of G' (Fig. 6.2 A). Addition of up to 20% collagen V caused a continuous decrease in G' , whereas G'' remained

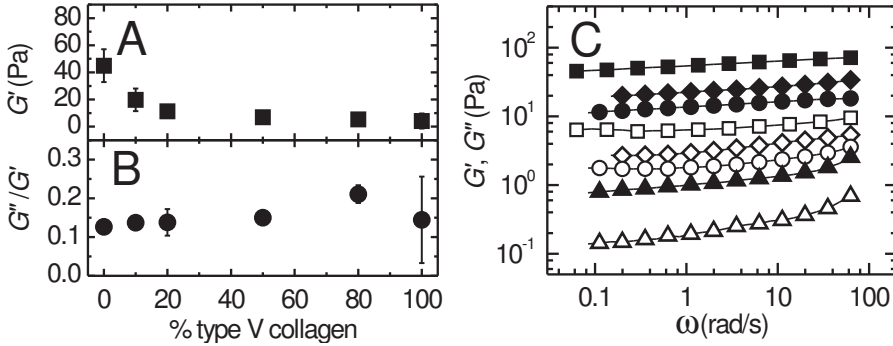


FIGURE 6.2. Effect of collagen type V percentage on the linear viscoelastic behavior of mixed collagen I/V networks at a fixed, total collagen concentration of 2 mg/ml. (A) Elastic modulus as a function of weight percentage collagen type V. (B) Corresponding loss tangent. (C) Frequency dependence of the linear elastic (closed symbols) and viscous (open symbols) shear moduli for collagen networks with varying collagen type I and V composition and a fixed total collagen concentration of 2 mg/ml. Symbols denote weight/weight ratios of 100/0 (squares), 90/10 (diamonds), 80/20 (circles), 0/100 (triangles). Data are presented as average \pm SD with $n=3$. Means were compared using unpaired Student's *t*-tests).

constant for larger collagen V contents (Fig. 6.2 A). The loss tangent, G''/G' , was around 0.13, independent of collagen V content (Fig. 6.2 B). Incorporation of collagen V did not influence the dynamics of stress relaxation in the collagen networks. G' was weakly dependent on frequency according to a power-law with an exponent of ~ 0.1 for all collagen compositions (Fig. 6.2 C). This type of behavior is typical for (bio)polymer gels that are transiently crosslinked by noncovalent interactions [297].

We next compared the dependence of the shear moduli on total collagen concentration for pure collagen I and hybrid collagen I/V (80/20) gels. In both cases, the stiffness increased when the concentration was raised from 0.5 to 3 mg/ml, but it saturated above 3 mg/ml to a value of 100 Pa for collagen I and 55 Pa for collagen I/V (Fig. 6.3 A). This saturation was not caused by incomplete fibrillization; concentration measurements on supernatants collected after precipitation of the fibrils by centrifugation showed less than 7% nonfibrillated collagen for all collagen concentrations (Fig. 6.4). The elastic moduli of pure collagen I networks are comparable to prior reports for rat tail collagen [243, 8] and pepsinized bovine skin collagen [297, 156, 322, 282, 283, 323, 294]. At the same total collagen concentration, the hybrid networks were always about 3-fold softer than pure collagen I networks (Fig. 6.3 C). The loss tangents of hybrid gels was similar to that of pure collagen I gels up to 3 mg/ml collagen, while at higher concentrations it was somewhat larger ($p < 0.05$, Fig. 6.3 B).

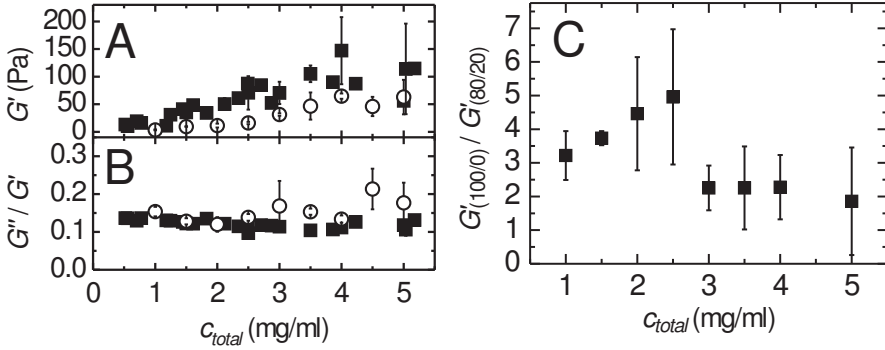


FIGURE 6.3. Effect of collagen V addition on the linear viscoelastic behavior of collagen networks. (A) Elastic moduli for pure type I collagen networks (solid squares) and mixed 80/20 collagen type I/V networks (open circles) as a function of total collagen concentration. (B) Corresponding loss tangents. (C) Ratio of the linear elastic modulus, G_0 , of collagen 100/0 and 80/20 as a function of total collagen concentration.

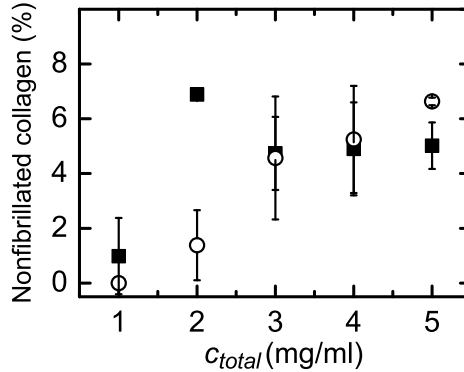


FIGURE 6.4. Percentage of nonfibrillated collagen versus total collagen concentration for type I (solid squares) and hybrid 80/20 collagen gels (open circles).

To assess whether collagen V affects the nonlinear elastic response of collagen networks, we subjected the gels to large amplitude oscillatory shear. Both collagen I networks and hybrid collagen I/V networks (80/20) stiffened up to collagen concentrations of 2 mg/ml and weakened at higher concentrations (Fig. 6.5 A), although the hybrid networks were overall softer. The critical strain where strain-stiffening set in for dilute gels was ca. 17% for both homotypic and heterotypic networks (Fig. 6.5 B). Consistent with prior reports for pepsinized collagen I [294], the critical strain was insensitive to collagen concentration. However, the pepsinized collagen gels stiffened over a concen-

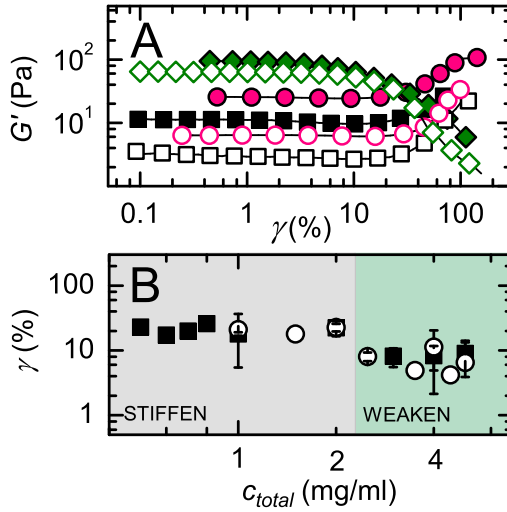


FIGURE 6.5. Effect of collagen V addition on the nonlinear rheology of collagen networks. (A) Both pure type I collagen networks (solid symbols) and hybrid 80/20 type I/V networks (open symbols) strain-stiffen for collagen concentrations of 1 mg/ml (black squares) and 2 mg/ml (pink circles), while they strain-weaken for larger collagen concentrations (green diamonds: 4 mg/ml). (B) Dependence of critical strain (onset of strain-stiffening, grey-colored area) and yield strain (onset of strain-weakening, green-colored area) on total collagen concentration for collagen I networks (solid squares) and hybrid collagen I/V networks (open circles).

tration range of 0.5-5 mg/ml, whereas rat tail collagen I gels studied here strain-weakened above 2 mg/ml. The yield strain characterizing the onset of strain-weakening was independent of collagen concentration, being around 8% for both homotypic and heterotypic gels (Fig. 6.5 B).

6.3.2 COLLAGEN NETWORK MICROSTRUCTURE

To investigate whether the effect of collagen V on collagen rheology is accompanied by structural changes on the network scale, we performed fluorescence confocal microscopy. Figure 6.6 (top panels) shows representative images of hybrid networks with varying mixing ratio but a fixed total collagen concentration of 2 mg/ml. The networks were rather homogeneous and isotropic for all collagen I/V ratios. Networks with 50% or 100% collagen V appeared to have somewhat larger open spaces than networks with 20% or less collagen V. This observation was confirmed by considering the average mesh size of the networks obtained by thresholding the images and measuring the distribution of distances between on-pixels (corresponding to detected fibers) [20]. For collagen gels containing predominantly collagen I (80-100%), the average mesh size was 3 μm . Networks containing 50% collagen V had an increased average mesh size of 4 μm , and networks composed of 100% collagen V had an even

larger average mesh size of 7 μm . To test whether collagen V affects the network structure at the fiber scale, we also performed atomic force microscopy (AFM) on 10x10 μm and 3x3 μm areas (Fig. 6.6, middle and bottom rows of images). The collagen fibers were smooth and appeared unbranched. Images of hybrid networks showed evidence of loose association of some of the fibrils into twisted bundles (white arrows in Fig. 6.6). Fibrils of pure type V collagen were thinner than the collagen I-containing fibers. Moreover, they were shorter, with a typical length of only 1 μm . In contrast, collagen I-containing fibers were too long to recognize both ends in 10x10 μm or 3x3 μm areas.

Pure collagen I and hybrid collagen I/V networks both displayed two distinct concentration regimes. They looked rather inhomogeneous with dense collagen areas interspersed with large open spaces at concentrations below 1 mg/ml, whereas they were more homogeneous above 2 mg/ml (Fig. 6.7, upper panels). In both concentration regimes, pure collagen I networks and hybrid networks of the same concentration looked comparable, indicating that collagen V did not substantially affect the network structure.

6.3.3 QUANTIFICATION OF FIBRIL DIAMETER

To quantify the effect of collagen V on fibril diameter, we performed light scattering measurements with turbidimetry as described in Chapter 2. The advantage of this approach is that it noninvasively measures the diameter and mass/length ratio of fibers in their hydrated state [52]. The average fiber mass/length ratio substantially decreased upon increasing the collagen V content from 0 to 100% (Fig. 6.6 A). Pure collagen I fibers had a mass/length ratio of $1.23 \cdot 10^{13}$ Da/cm. Given a quarter-staggered axial packing of collagen molecules with a periodicity of $D = 67.2$ nm (Fig 6.9 B), the number of molecules per cross-section is $N = \mu/(M/4.6D)$, where M is the molecular mass (290 kDa for collagen I and 352 kDa for collagen V) [139]. Pure collagen I fibers thus had close to 1200 monomers per cross-section (Fig. 6.8 A). Fibrils with 10% collagen V had a comparable mass/length ratio (N around 1700), but incorporation of 20% or more collagen V caused a reduction in μ compared to pure collagen I fibrils. Pure collagen V fibrils had a 5-fold lower mass/length ratio than pure collagen I fibrils ($p < 0.01$) with $\mu = 2.3 \cdot 10^{13}$ Da/cm and $N = 220$.

The diameter of the fibers as measured with turbidimetry also decreased with increasing collagen V content, but only weakly, going from 117 nm at 0% collagen V to 96 nm at 50% collagen V (Fig. 6.6 B). Surprisingly, the apparent diameter of pure collagen V fibrils was 157 nm, larger than that of pure collagen I fibrils. However, this diameter value is probably inaccurate because the light scattering spectra for collagen V were not well-fitted by the theoretical expression for rigid fibrils (see Chapter 2), Fig. 2.3. The reason is probably that the theoretical analysis is only valid when the fiber length exceeds 800 nm, which is close to the average length of the pure collagen V observed by AFM [327]. We note that the measured diameters for all collagen compositions are larger than expected based on the mass/length ratio assuming a close-packed lateral packing arrangement of tropocollagen monomers in the fibers. For a close-packed arrangement, the effective diameter should be $d_{eff} = 1.83\sqrt{N}$ (nm)

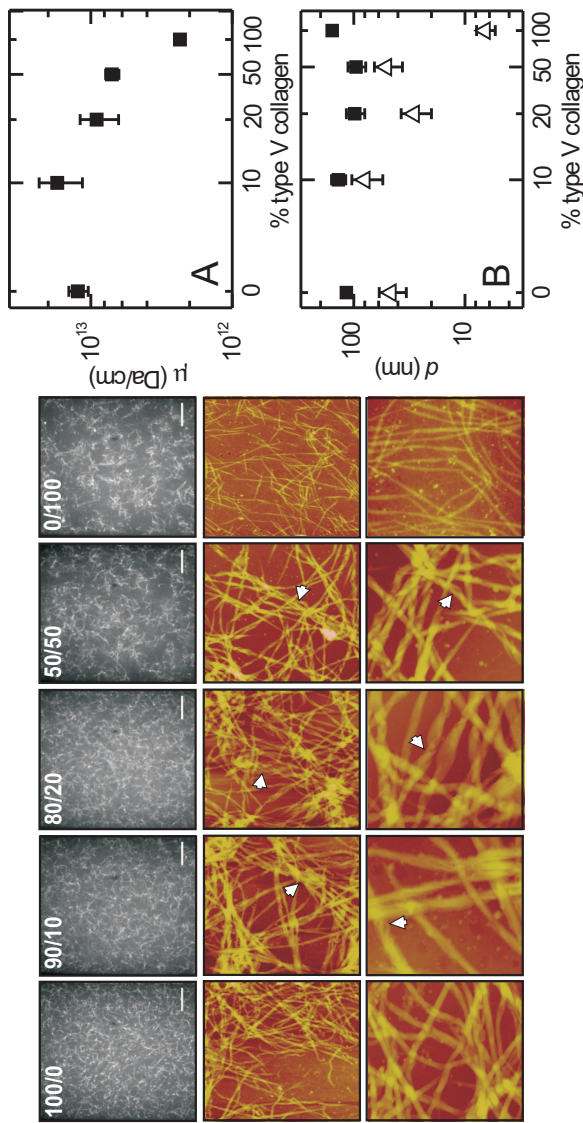


FIGURE 6.6. Effect of collagen V on network architecture and fiber structure in networks with a constant overall collagen concentration of 2 mg/ml. (Top row of images): Confocal micrographs of hydrated collagen networks with different collagen type V contents as indicated. The fibers are labeled with a fluorescent collagen-binding protein, AlexaFluor488-CNA35. Images represent single confocal planes. Bars, 10 μ m. (Middle row of images): AFM images of dried collagen fibers deposited on mica, showing 10x10 μ m areas. (Bottom row of images): AFM images of 3x3 μ m areas. Arrows point to loose bundles of fibrils. (A) Fibril mass/length ratio measured with turbidimetry, versus collagen V content. (B) Dependence of average fiber diameter on collagen V content, obtained for hydrated fibrils with turbidimetry (closed squares) and for dried fibrils with AFM (open triangles).

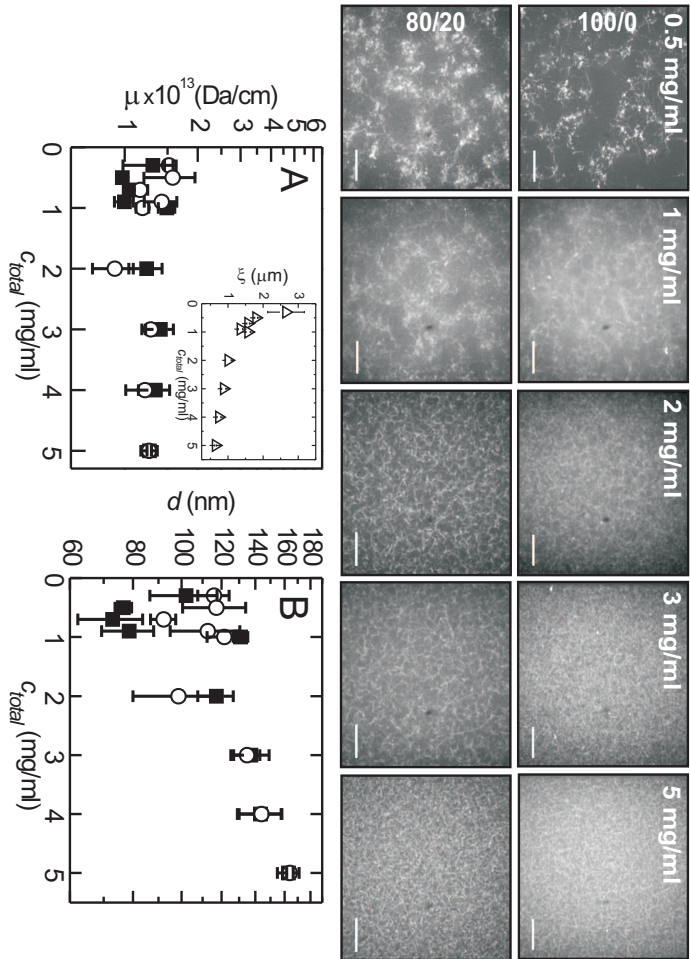


FIGURE 6.7. Effect of total collagen concentration on network architecture and fiber diameter for pure type I and hybrid (80/20) I/V collagen networks. *Top row of images:* Confocal micrographs of pure collagen I networks with concentration as indicated. *Bottom row of images:* Corresponding confocal images of hybrid collagen networks. Images are maximum intensity projections of z-stacks of 101 planes over a depth of 10 μm . Bars, 10 μm . (A) Fiber mass/length ratio and (B) diameter for pure type I networks (solid squares) and 80/20 I/V networks (open circles) measured on hydrated collagen gels by turbidimetry. Inset in (A) shows the concentration dependence of the network mesh size that is expected for pure type I collagen networks based on the known collagen mass concentration and measured fiber mass/length ratio, assuming a homogeneous fiber distribution.

[139]. Fig. 6.9 shows that the measured diameters do scale as \sqrt{N} , but the absolute values are larger than the predicted values by a factor 2. This difference suggests that the fibers are more open than regularly packed fibers, which is compatible with the loose bundling seen in the AFM images (Fig. 6.6, image panels).

For comparison, we also measured the fiber diameter from the apparent height observed by AFM (Fig. 6.6 B). In agreement with the turbidity data, AFM imaging also demonstrated a reduction in fiber diameter with increasing collagen V content, but this reduction was stronger than the reduction observed with turbidimetry. All height values were lower than the diameter values measured by turbidimetry. This may reflect shrinkage of the fibers imaged by AFM during drying. Furthermore, turbidimetry may measure a larger average diameter due to scattering contributions from bundles of collagen fibrils. For pure type V fibrils, AFM gave a substantially lower diameter value than turbidimetry, namely ~ 7 nm. Our data are consistent with prior electron microscopy (EM) studies [3, 28, 56], which reported a decrease of fiber diameter from ~ 150 nm for pure collagen I to 25-40 nm for pure collagen V. However, a direct quantitative comparison is difficult since both AFM and EM probably suffer from (different degrees of) fiber shrinkage due to drying, and since fiber bundles contribute to light scattering in a way that is difficult to model theoretically.

To evaluate the dependence of fiber diameter on collagen concentration, we performed turbidimetry on gels of varying total concentration but fixed collagen V content (0% or 20%). The average mass/length ratio of the fibrils was similar in both types of networks and did not show a clear dependence on collagen concentration. On average, the mass/length ratio was around $1.2 \cdot 10^{13}$ Da/cm (Fig. 6.7 A), corresponding to ~ 1300 molecules/cross-section (Fig. 6.8 B). The diameter of the fibrils showed two distinct concentration regimes. At low collagen concentrations (0.3-1 mg/ml), the diameter of the fibers showed large, inconsistent variations with collagen concentration (Fig. 6.7 B). The diameter varied from 76 to 130 nm for pure collagen I fibers, and from 90 to 120 nm for the hybrid fibers. These large variations may reflect the heterogeneous structure of the low-density networks visible by confocal microscopy. At high collagen concentrations (2-5 mg/ml), where the network structure is more homogeneous, the fiber diameter showed a progressive increase with increasing collagen concentration. The diameter of pure collagen I fibers increased from 117 to 163 nm, and the diameter of hybrid fibers increased from 100 to 164 nm (increases significant with $p < 0.001$). The increase in fiber diameter between 2 and 5 mg/ml collagen seems contradictory with the constant mass/length ratio in this concentration regime. A possible explanation for this apparent contradiction is lateral aggregation of the fibrils, which has also been reported in prior EM and confocal studies [323, 317]. Our turbidity measurements are consistent with loose lateral bundling, since the measured diameters are consistently larger than the values expected for fibers with a regular lateral packing (Fig. 6.9).

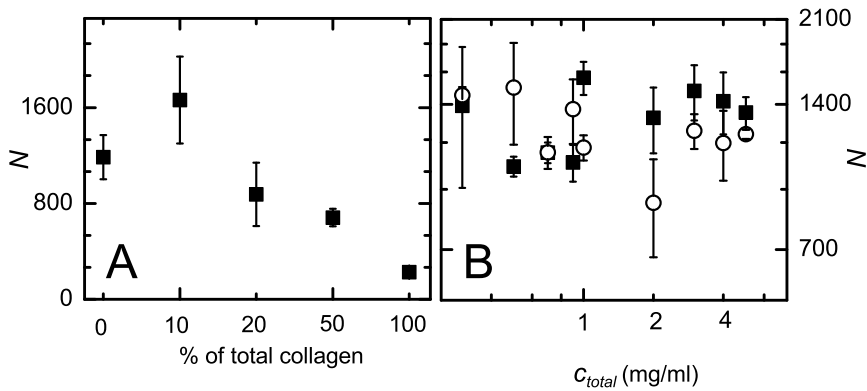


FIGURE 6.8. Number of collagen monomers per cross-section N , calculated from mass/length ratios measured by turbidimetry, assuming a D-periodic axial packing. (A) Dependence of N on collagen V content for mixed collagen I/V networks at a constant total collagen concentration of 2 mg/ml. (B) Dependence of N on total collagen concentration for pure collagen I networks (solid squares) and for mixed 80/20 collagen I/V networks (open circles).

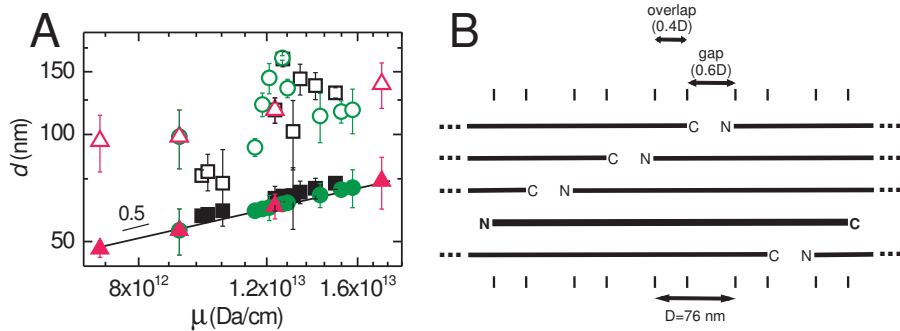


FIGURE 6.9. (A) Diameter of hydrated fibrils measured by turbidimetry (open symbols) and expected diameter calculated from the measured mass/length ratio (closed symbols), assuming regular axial and lateral packing, where $d = 1.83\sqrt{N}$ and N is the number of molecules per cross-section. Data are plotted versus the mass/length ratio measured by turbidimetry. Symbols denote: pure collagen I networks (squares), collagen I/V 80/20 networks (circles) and networks with different I/V ratio (triangles). Solid line indicates expected \sqrt{N} scaling. (B) Cartoon showing axial packing of collagen fibers.

6.4 DISCUSSION

The main finding in this chapter is that collagen V addition strongly decreases the elastic shear modulus of collagen I gels. Incorporation of 10% collagen V decreased the stiffness 2-fold, while 20% collagen V reduced the stiffness 3-5 fold. Pure collagen V networks were 10-fold softer than pure collagen I networks. Fluorescence microscopy showed no major change in network microstructure in the presence of collagen V. Turbidimetry showed that hybrid fibers had a

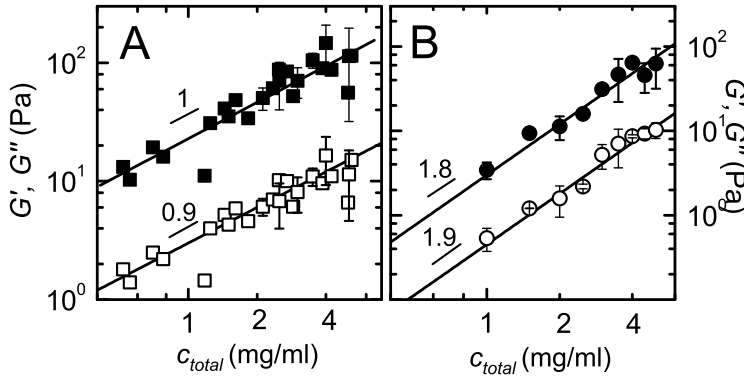


FIGURE 6.10. Linear elastic (solid symbols) and viscous (open symbols) shear moduli for (A) pure type I collagen networks and (B) mixed 80/20 collagen type I/V networks. Lines indicate power-law fits with exponent as indicated.

smaller mass/length ratio and somewhat smaller diameter than pure collagen I fibrils. AFM confirmed that the fibers became thinner with increasing collagen V content, and showed that pure collagen V fibers were shorter than collagen I containing fibers. Moreover, AFM images showed evidence of a tendency for collagen fibers to form loose bundles.

To interpret the effect of collagen V on network stiffness, in terms of the structural properties of the networks, we will compare the measured concentration dependence of the network stiffness (Fig. 6.3 A) with theoretical models for stiff biopolymers. There are two distinct models, *thermal* models based on entropic elasticity and *athermal* models based on enthalpic elasticity. In both cases, one needs to further distinguish between nonaffine and affine regimes. Computer simulations predict that sparsely crosslinked networks will deform in a nonuniform (*nonaffine*) manner [118, 311, 72, 144, 49, 67]. There are no simple analytical models in this regime. Densely crosslinked networks will deform *affinely*, resulting in fiber stretching. In this regime, the network elasticity can be calculated analytically from the number density of the fibers and their stretching rigidities [285].

Affine models predict power law dependencies of the linear elastic modulus, G_0 , on fiber concentration, according to $G_0 \sim c^{11/5}$ for a thermal sys-

tem [195] and $G_0 \sim c^1$ for an athermal system [119, 49]. A log-log plot of our data suggests power-law scaling with exponents of 1.0 ± 0.1 for pure type I gels and 1.8 ± 0.1 for 80/20 collagen I/V networks (Fig. 6.10). However, when plotted on linear scales (Fig. 6.3 A), the data actually show two distinct concentration regimes: below 3 mg/ml the stiffness increases with concentration, but above 3 mg/ml it is constant. The simple scaling predictions assume a constant fiber diameter. We showed that the fiber diameter changes with concentration (Fig. 6.7 B). This will affect the bending and stretching rigidity of the fibers, as well as the scaling of fiber density with protein concentration. Therefore, we do not expect a simple scaling law for $G_0(c)$. Prior studies of collagen rheology [323, 294, 297, 156, 282, 283] reported widely varying power law exponents ranging from 1 to 3. Our observations suggest that this variability may be, at least in part, related to different concentration dependencies of the fibril diameter, which will likely depend on the collagen source, presence of telopeptides, and assembly conditions.

Treating the fibers as homogeneous elastic cylinders of radius r and Young's modulus E , the bending rigidity is $\kappa_{bend} = \pi E r^4 / 4$ and the stretching rigidity is $\kappa_{stretch} = \pi E r^2$. The length density of fibers per volume, ρ , follows from the fiber mass/length ratio and weight concentration of collagen as $\rho = c N_A / \mu$, assuming randomly arranged fibers, where N_A is Avogadro's constant. The mesh size, $\xi = \rho^{1/2}$, should decrease from 2.7 μm at 0.3 mg/ml collagen to 0.6 μm at 5 mg/ml (open triangles in inset of Fig. 6.7 A). This prediction is in qualitative agreement with the confocal images, at least above 2 mg/ml collagen, where the networks are rather homogeneous. At lower concentrations, the images show network inhomogeneities, so that the calculation underestimates the mesh size (below, we neglect this effect). Note that the calculated mesh size at a collagen concentration of 2 mg/ml is smaller than the mesh size obtained by binarizing the confocal images (1 μm compared to 3 μm), which may reflect network inhomogeneities. However, the optical mesh size also has a sizeable error, on the order of 1 μm , and is sensitive to the threshold value chosen for binarizing the images. We therefore treat this parameter as indicative of changes in response to changes in collagen concentration, but do not use it in calculations of network elasticity below.

If the network is composed of filaments with appreciable thermal bending, the network elasticity can be calculated from the entropic resistance of polymer segments between crosslinks to stretch. This approach has been previously applied to collagen gels [243, 323]. The linear elastic modulus is $G_0 = 6 \rho k_B T l_p^2 / l_c^3$, where k_B is Boltzmann's constant, T is absolute temperature, and l_p is persistence length, which is related to the bending rigidity as $l_p = \kappa_{bend} / k_B T$ [195]. The crosslink distance, l_c , is proportional to the entanglement length, l_e , and related to ρ and l_p by the scaling relation [195]: $l_c = A l_p^{1/5} \rho^{-2/5}$, where A is unknown but empirically found to be close to 1 (see Chapter 3). Treating the fibers as homogeneous elastic beams with $E = 70$ MPa (a value chosen to be consistent with two prior studies of collagen gel rheology [282, 260, 283] and consistent with single fibril measurements, as summarized in the Table 1 in the Appendix) and a diameter equal to the average value measured by turbidimetry, we obtain $l_p = 15$ cm at 2 mg/ml collagen and $G_0 = 452$ kPa, which is

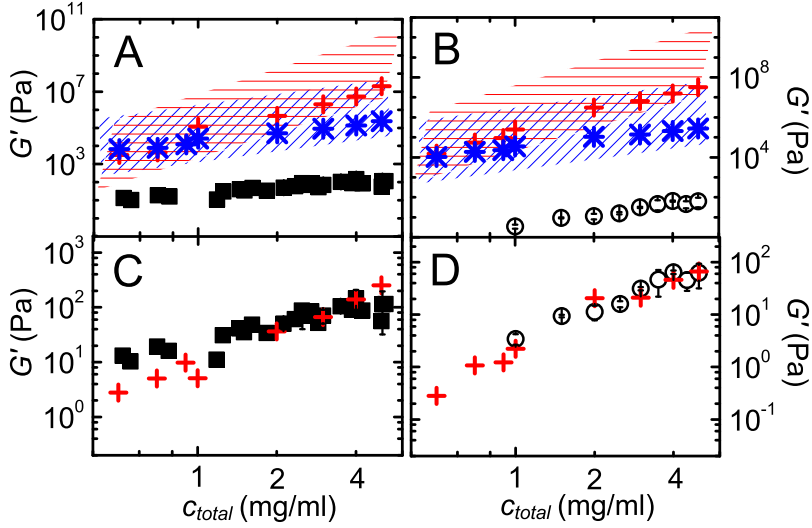


FIGURE 6.11. Experimental data for collagen network stiffness as a function of total collagen concentration, compared with theoretical estimates. Collagen fibers are modeled as simple elastic beams with a diameter measured by turbidimetry and a Young's modulus of 70 MPa. (A) Squares represent experimental data for a pure collagen I network. Crosses show the affine entropic model prediction, assuming that the network compliance is dominated by the thermal (entropic) compliance of collagen fibers when stretched. Stars represent the affine mechanical model prediction, assuming that the network compliance is dominated by mechanical (enthalpic) stretching of collagen fibers. Shaded areas behind symbols show predicted, from affine entropic and affine enthalpic model, range of elastic modulus based on a Young's modulus in the range of 30-800 MPa. (B) Affine entropic and affine mechanical prediction for collagen 80/20 networks (open circles represent experimental data). (C) Affine entropic model (crosses), using the fiber persistence length as a free parameter, with $l_p = 180 \mu\text{m}$ for collagen type I networks and (D) $l_p = 70 \mu\text{m}$ for hybrid 80/20 gels.

10^4 times larger than the measured modulus (Fig. 6.11 A). Even if we allow for variations in fiber diameter (over a range of 100 to 160 nm as measured by turbidimetry) or Young's modulus (over the range of 30-1500 MPa reported in the literature), we cannot account for this large discrepancy. However, this estimate disregards the complex internal structure of the fibers. If we instead treat l_p as a free parameter to account for fiber softening due to molecular defects such as kinks [204], slippage between fibrillar subunits [273, 204, 45], or lateral inhomogeneities in molecular packing [113], we can only get agreement between our data and the model if we assume $l_p = 180 \mu\text{m}$ for pure collagen I (Fig. 6.11 C) and $l_p = 70 \mu\text{m}$ for hybrid (80/20) networks (Fig. 6.11 D). These values are many orders of magnitude smaller than anticipated based on the bulk

Young's modulus. Interestingly, the numbers are closer to values reported in a recent EM study, where a persistence length of 10 μm was inferred from the shape of collagen fibrils deposited and dried on an EM grid [274]. There are two alternative interpretations of this intriguing finding. One interpretation is that the fibrils are indeed much more flexible than anticipated based on continuum elastic calculations due to their complex internal structure. A minimum limit on the persistence length can be estimated by modeling the collagen fibril as a bundle of fully decoupled tropocollagen molecules [61]. This yields an estimate of 17 μm for fibrils with 1200 molecules per cross-section, each having a persistence length of 14.5 nm [288]. However, collagen fibrils look very straight in all our AFM and optical microscopy (bright field) images. An alternative explanation for the short l_p reported from the EM study is that it reflects intrinsic curvature rather than thermal fluctuations. This interpretation was also put forward by A. Stein and D. Vader et al in their rheology study [282, 283], where experimental data were well-fit by a computational 3D-fiber model assuming that the fibers are rigid rods (having no thermal fluctuations), but with an intrinsic curvature characterized by a geometrical persistence length of 20 μm . We favor this second explanation, and believe that it is unlikely that collagen network elasticity is entropic in origin. However, a definitive test will be needed in the form of direct dynamic measurements of the filament persistence length in solution. This can be achieved by measuring thermal bending undulations of the fibers, e.g. by optical tweezers [232].

If we assume instead that the fibers behave as stiff rods, the network elasticity should be enthalpic in origin and reflect the mechanical resistance of the fibers to axial stretch. The elasticity of an isotropic network of rods is $G' = (1/15)\rho\kappa_{stretch}$. At 2 mg/ml collagen (where the fiber diameter is 117 nm), $\kappa_{stretch}$ is 753 nN and G' is 50 kPa, which is 1000-fold larger than the measured stiffness (Fig. 6.11 A). This large discrepancy suggests that the collagen networks do not deform affinely at the fiber scale. Any nonaffine deformation such as fiber bending and reorientation will reduce G' relative to the affine limit [311, 118, 144, 67, 282, 283, 135, 40]. It is difficult to calculate the impact of nonaffinity on the rheology since G' will be sensitive to the exact network connectivity, which is determined by collagen fiber length and the topology and mechanical properties of the noncovalent (physical) crosslinks between fibers, which are all unknown. A more promising approach is to simulate 3D-fiber networks using collagen-based parameters as input [49, 144, 67, 282, 283].

Our nonlinear rheology data provide circumstantial evidence of nonaffinity. We showed that networks up to 2 mg/ml collagen strain-stiffen. In networks of stiff rods, strain-stiffening is indeed expected when the elastic energy is predominantly stored in fiber bending. The reason is that large shears align the filaments, inducing a transition to a stiffer elastic regime dominated by fiber stretching [224, 67]. Numerical simulations also suggest that nonaffine strain-induced alignment explains the nonlinearity of collagen networks [282, 283]. Interestingly, we observed that networks of more than 2 mg/ml collagen strain-weaken. We speculate that the thicker fibers formed in this regime are too stiff to bend, forestalling a bending-to-stretching transition. We plan to measure nonaffinity directly by mapping the 3D-strain field of sheared collagen gels by

confocal microscopy. Prior microscopy studies suggest nonaffinity [260, 250, 54]. Further evidence for nonaffinity was presented in the form of a system size dependence of the nonlinear rheology [8].

There is currently no simple analytical expression to predict the elastic modulus of networks of stiff polymers that deform nonaffinely. This hampers a quantitative interpretation of the dependence of collagen network stiffness on collagen V content. Collagen V does not appear to substantially modify the network architecture. Nevertheless, it is conceivable that collagen V changes intermolecular interactions between tropocollagen monomers. Collagen fibrils have a complicated internal architecture, involving laterally associated microfibrils, that themselves are built up of tropocollagen subunits that are noncovalently bonded. It is conceivable that incorporation of collagen V alters the intermolecular spacing and/or intermolecular adhesion, thereby influencing the shear strength between adjacent molecules and the bending rigidity. This could be tested by bending or stretching tests on single fibers [273, 321, 296, 271]. The molecular adhesion between collagen I and V molecules could be tested by osmotic pressure measurements coupled with X-ray scattering [170]. Alternatively, collagen V may alter interfibrillar interactions and thus the network connectivity. Our AFM images showing loose bundling of hybrid fibrils suggest that the hybrid fibers may be more adhesive than pure collagen I or pure collagen V fibrils. Since the elasticity of the collagen networks is nonaffine in origin we cannot easily distinguish between effects of collagen V on the fiber or network level. Multiscale simulation models of collagen networks taking into account collagen's remarkable hierarchical structure may potentially facilitate a molecular interpretation of the influence of co-polymerizing agents such as collagen V on network mechanics [45].

Collagen V's effect on the rheology of collagen networks may play an important role in soft connective tissues *in vivo*. Most healthy adult tissues contain only 2-5% collagen V [202], but the collagen V level is higher in embryonic and regenerating tissues [255, 35]. Moreover, several connective tissue diseases [197, 7, 308] and pathological conditions such as tumors, atherosclerotic plaques, and scars [35] are associated with anomalous levels of collagen V. Our work suggests that collagen V influences collagen matrix mechanics. This may in itself adversely affect tissue function, but it may also influence the behavior of the resident cells. It has been shown that cell adhesion, cytoskeletal structure, and even differentiation are strongly influenced by the stiffness of the extracellular collagen matrix [328, 75]. *In vitro* studies revealed several effects of collagen V on cell behavior: collagen V enhances collagen gel contraction by fibroblasts [21] and affects cell migration [35, 198, 238, 6, 58]. It has been suggested that differences in integrin-mediated cellular interactions with collagen might be involved [21]. Since our findings show that collagen V can influence collagen network mechanics, it is conceivable that collagen V may also influence cell behavior by causing mechanical changes in the extracellular matrix. However, to test this hypothesis, it would be necessary to measure effects of changing collagen V levels on the mechanical properties of biological tissues. The effect of collagen V on tissue mechanics *in vivo* may namely be different from its effect *in vitro*. Collagen V *in vivo*, for instance, retains long propeptides, in contrast

to the atelocollagen V used in this study, whose effect on collagen rheology is yet unknown. Furthermore, collagenous tissues contain many non-collagenous components such as proteoglycans and glycoproteins, which may modulate the overall mechanical response.

6.5 CONCLUSION

We discovered that incorporation of collagen type V substantially lowers the stiffness of reconstituted networks of collagen type I over a wide range of total collagen concentrations. Homotypic and heterotypic collagen networks both display two distinct concentration regimes, differing in structural organization and mechanical behavior. At low concentrations (1 mg/ml or less), the networks are somewhat heterogeneous, strain-stiffen, and the plateau modulus increases with increasing collagen concentration. In contrast, at high concentrations (more than 2 mg/ml), the networks are more homogeneous and strain-soften and the plateau modulus is concentration-independent. A direct comparison of the rheology data with theoretical predictions for stiff polymers suggests that the network elasticity is dominated by nonaffine deformation modes. Unfortunately, there is no tractable analytical expression available which predicts the network modulus in this mechanical regime, hampering a quantitative interpretation of the influence of collagen V. Collagen V may potentially affect the intermolecular interactions among tropocollagen monomers, which may in turn affect the rheology by changing the rigidity of the fibrils as well the interactions among fibrils. The effect of collagen V on network stiffness is relevant to interpret the role of changed collagen V levels in fetal development and natural tissue regeneration as well as in various connective tissue disorders. Moreover, our findings suggest that collagen composition can be used as a powerful control parameter to design collagen-based biomaterials with controlled mechanical properties.

6.6 APPENDIX

6.6.1 SUMMARY OF YOUNG'S MODULI (E) AND PERSISTENCE LENGTHS (l_p) DETERMINED FOR HYDRATED COLLAGEN FIBRILS

TABLE I. Summary of Young's moduli (E) and persistence lengths (l_p) determined for hydrated collagen type I fibrils from various sources, using various experimental techniques. Persistence length values with asterisk are calculated from E and the fiber diameter, d , assuming homogeneous and linear elasticity.

E , mean (range) (MPa)	l_p	D (nm)	Method	Collagen source	Ref.
32	0.004-0.03 cm*	10-30	AFM ¹ stretching	Reconstituted from collagen produced by fibroblasts	[103]
70-170	493-6421 cm*	280-426	AFM bending	Bovine tendon	[321]
250-450	459-826 cm*	~200	AFM stretching	Bovine tendon	[296]
470 (110-1470)	952-21713 cm*	205-448	MEMS ² stretching	Sea cucumber dermis	[271]
1.2	0.03-0.7 cm*	70-150	Fibril indentation	Bovine tendon	[105]
500	573428 cm*	1000	Fiber stretching	Rabbit patellar tendon	[205]
35 kPa*	10 μ m	50-500	Fiber shape analysis	Bovine skin	[274]

¹ Atomic Force Microscopy

² MicroElectroMechanical System

* Calculated assuming continuum elasticity, and an isotropic cylindrical rod

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